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<p>(21) International Application Number: PCT/US99/09784</p> <p>(22) International Filing Date: 5 May 1999 (05.05.99)</p> <p>(30) Priority Data: 60/098,712 1 September 1998 (01.09.98) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, Madison, WI 53705 (US).</p> <p>(72) Inventor; and</p> <p>(75) Inventor/Applicant (<i>for US only</i>): THOMSON, James, A. [US/US]; Wisconsin Regional Primate Research Center, 1223 Capital Court, Madison, WI 53715 (US).</p> <p>(74) Agent: SCHWARTZ, Carl, R.; Quarles & Brady LLP, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: PRIMATE EMBRYONIC STEM CELLS WITH COMPATIBLE HISTOCOMPATIBILITY GENES</p> <p>(57) Abstract</p> <p>Disclosed herein are methods for producing primate embryonic stem cells that are MHC-matched to a specific donor individual. In one aspect, one transplants donor primate cell nucleus to an enucleated primate oocyte. The culture of the resulting nuclear transfer product to the blastocyst stage and the subsequent isolation of primate ES cells from the inner cell mass is designed to allow the production of ES cells that are genetically identical for all nuclear genes to the original donor of the nucleus. Differentiated cells derived from the primate ES cells are less likely to be rejected by the donor's immune system when used for therapeutic purposes such as transplantation.</p>			

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18063

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C07H 21/04; C12N 5/00, 5/06, 5/10, 5/12, 5/16, 5/22, 5/26, 5/28, 15/02, 15/07, 15/08, 15/09, 15/11, 15/12, 15/16, 15/18, 15/19, 15/52

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/325, 350, 351, 353, 354, 363, 364, 366, 368, 369, 370, 371, 372, 372.1, 372.2, 372.3, 375, 377, 455; 536/23.1, 23.2, 23.5, 23.51

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

435/325, 350, 351, 353, 354, 363, 364, 366, 368, 369, 370, 371, 372, 372.1, 372.2, 372.3, 375, 377, 455; 536/23.1, 23.2, 23.5, 23.51

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, MEDLINE, EMBASE, BIOSIS, INPADOC, CAPLUS

search terms: cytoplasm, donor, recipient, undifferentiated, dedifferentiated, oocyte, embryonic, somatic, cytoplasmic transfer, nuclear transfer, telomerase, life-span, hormone, growth factor, enzyme, agonist, antagonist, antibody, clotting, antibacter?, antiviral, cytokine, embryonic stem

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-16 and 20-23, drawn to a method for reprogramming and/or altering the life-span of a desired cell and compositions obtained by the method.

Group II, claim(s) 17-19, drawn to an improved method of gene therapy.

Group III, claim(s) 24-25, drawn to an improved method of cloning a non-human mammal via nuclear transfer.

Group IV, claims 26-32, drawn to a method for producing a culture comprising embryonic stem cells and compositions obtained by the method.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the methods of each of Groups I-IV are directed to producing different end products or end results, and require different reagents, different methods steps, and different technical considerations. For example, technical considerations associated with gene therapy protocols of Group II are not necessary to reduce to practice the methods of Groups I, III, or IV. As another example, the method steps of Group III require introduction of a whole cell or nucleus into an enucleated oocyte, i.e., steps and reagents that are not required to reduce to practice the methods of Groups I, II, or IV. As the methods of Groups I-IV are distinct, each from the other, the inventions listed as groups I-IV lack the same or corresponding technical features.

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EE	Estonia						

PRIMATE EMBRYONIC STEM CELLS WITH COMPATIBLE
HISTOCOMPATIBILITY GENES

CROSS-REFERENCE TO RELATED APPLICATION

Not applicable.

5 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with United States government support awarded by the following agencies: NIH Grant Nos: RR11571; RR/AG00167; HD34215 and NIH Grant No: RR12804. The United States has certain rights in this

10 invention.

BACKGROUND OF INVENTION

The present invention relates to production of primate embryonic stem (ES) cells that are MHC-matched to a specific donor individual. More particularly, it

15 relates to ES cells that are designed to be genetically identical for all nuclear genes to the donor.

Primate embryonic stem cells have been developed which are capable of unlimited undifferentiated proliferation while maintaining the developmental potential to form any cell type of the body. See U.S. Ser. No. 08/591,246 filed January 18, 1996 (issue fee paid), J. Thomson *et al.*, 92 P.N.A.S. USA 7844-7848 (1995), J. Thomson *et al.*, 55 Biol. Reprod. 254-259 (1996), J. Thomson *et al.*, 38 Curr. Top. Dev. Biol. 133-25 (1998), and J. Thomson *et al.*, 106 AMPHIS 149-157 (1998). The disclosure of these publications, and of all other publications referred to herein, are incorporated by reference as if fully set forth herein.

Such cells derived from undifferentiated primate ES cells were in part developed to provide source material for transplantation therapies. However, a potential problem with ES cell-based transplantation therapies is 5 the possibility of immune rejection of the transplanted cells. While there are a number of drugs that suppress immune rejection responses, they typically have side effects and significant cost. It is preferable to provide transplantation therapies which minimize or 10 eliminate the need for such drugs.

The ability of the oocyte cytoplasm to reprogram nuclei from differentiated cells differs significantly between vertebrate taxa. Oocytes from ungulates as a group (including cows and sheep) have the ability to 15 reprogram nuclei from fetal cells. See J. Cibelli *et al.* 280 Science 1256-1258 (1998), A. Schnieke *et al.*, 278 Science 2130-2133 (1997), and I. Wilmut *et al.*, 385 Nature 820-813 (1997).

There is also a report of the cloning of an adult 20 sheep ("Dolly") from an adult sheep nucleus. See I. Wilmut *et al.*, 385 Nature 820-813 (1997).

There have been recent reports regarding successful techniques for cloning mice. See e.g. T. Wakayama *et* 25 *al.*, 394 Nature 369 (1998). There was also a report of an attempt to produce a rhesus monkey by nuclear transfer (L. Meng *et al.*, 57 Biol. Reprod. 454-459 (1997)). The authors used undifferentiated blastomere nuclei from preimplantation embryo as the nuclear donors. However, the donor nuclei were from undifferentiated cells.

30 Changes in the status of imprinting genes occur spontaneously in cells in tissue culture, and there are

likely to be random changes in the status of imprinted genes in specific differentiated cells during normal development. These changes in the status of imprinted genes are incompatible with normal development, so a fetus derived from a nuclear donor with alterations in the status of imprinted genes is much more likely to abort. The random drift in the status of imprinted genes is one likely explanation for the high rate of gestational failure of nuclear transfer experiments using nuclei from differentiated cells.

In WO 98/07841, there was a report of the derivation of a clump of "cells" by virtue of implanting a human nucleus into an enucleated ungulate oocyte. Although the PCT refers to these "cells" as "stem cell-like", no evidence was provided that these were viable cells, (instead of cell fragments), no evidence was provided that they actually contained human nuclei (no karyotype was performed), and no evidence was provided that they had any defining characteristics of stem cells (e.g. capable of prolonged undifferentiated proliferation, capable of differentiation of the three embryonic germ layers).

Indeed, other reports clearly demonstrate that such interspecies nuclear transfer produced cells would not be viable because of a complete functional incompatibility between ungulate mitochondria and human nuclei. See L. Kenyon et al., 94 P.N.A.S. USA 9131-5 (1997). The PCT publication itself noted that they did not try to produce an ES cell colony from their 4 to 16 cell stage structures. While the publication speculated that it might be possible to transfer human cell nuclei into

human oocytes using similar methods, it gave no examples or description of what would need to change to make that work. Moreover, their apparently poor results with respect to even hybrids would indicate otherwise.

5 Thus, it can be seen that a need exists for techniques of developing stable primate embryonic stem cells which contain histocompatibility genes (preferably all nuclear genes) identical to those of selected donors.

BRIEF SUMMARY OF THE INVENTION

10 In one aspect the invention provides a primate embryonic stem cell that is at least MHC-compatible to a selected donor. The cell has been derived by transplantation of cell nucleus from a differentiated cell of the donor to an enucleated primate oocyte. The 15 concept is for use with a wide variety of primates such as macaques, marmosets and humans.

20 "Derived" is used here, and in the rest of this patent, in its broadest sense. It includes both direct derivation as well as indirect derivation. By "MHC-compatible" I mean that the cell has a major histocompatibility complex that matches that of the selected donor.

25 In another aspect the invention provides a purified preparation of primate (preferably human) embryonic stem cells which (i) is capable of proliferation in an in vitro culture, (ii) maintains the potential to differentiate to derivatives of endoderm, mesoderm, and ectoderm tissues in the culture, (iii) is inhibited from differentiation when cultured on a fibroblast feeder 30 layer, and (iv) has a major histocompatibility complex from a foreign source (e.g. one derived from a donor).

In a preferred form the cell is genetically identical with respect to all nuclear genes to a specified nuclear donor.

Unlike intact, whole mammals, ES cell are viable
5 with changes in the status of imprinted genes.

Therefore, the derivation of ES cells by the transfer of differentiated cell nuclei to an enucleated oocytes should allow the formation of histocompatible ES cells even when the nucleus is from a cell that would not allow
10 the cloning of an intact adult primate.

In still another aspect of the invention a method is provided for creating the aforesaid cells wherein the cells are derived by transplantation of cell nucleus from a differentiated cell of the donor to an enucleated
15 primate (preferably human) oocyte.

By reprogramming the nucleus of a differentiated cell from a specific individual to an ES cell nuclear state, it is possible to make ES cells that are MHC-matched to the specific individual. So, for example, one
20 can take a skin fibroblast or other cell from a patient with juvenile onset diabetes, reprogram the differentiated nucleus by transfer to an enucleated oocyte, culture the nuclear transfer product to the blastocyst stage, derive undifferentiated ES cells from
25 the inner cell mass, differentiate the ES cells to pancreatic β -cells, and use those β -cells for transplantation therapy to treat the diabetes in the same way that whole pancreas transplants are currently used for this purpose (albeit without the need for
30 immunosuppressive drugs).

The reprogrammed cells would be genetically matched for all nuclear genes of the nuclear donor including genes of the major histocompatibility complex (MHC).
5 Immune rejection of these cells by the individual that donated the nucleus is therefore much less likely.

It is also proposed that the MHC-matched ES cells can be formed by fusion between differentiated cell karyoplasts from a donor and primate ES or primate embryonal carcinoma (EC) cell cytoplasts.

10 Human embryonal carcinoma (EC) cells are the stem cells of teratocarcinomas. EC cells are essentially the malignant equivalent to ES cells, and some human EC cell lines maintain the potential to form all three embryonic germ layers.

15 Because human EC cells are highly aneuploid (lack a normal complement of chromosomes), they have a significantly more restricted developmental potential than ES cells. However, their cytoplasm should have the same required factors to reprogram the nucleus from a
20 differentiated cell, and if that nucleus is euploid (has a normal complement of chromosomes) the EC cell cytoplasm-differentiated cell karyoplast fusion product should have a greater developmental potential than the original EC cell.

25 The objects of the present invention therefore include providing:

- (a) primate embryonic stem cells with MHC-compatibility to selected donors;
- (b) primate embryonic stem cells derived from
30 enucleated oocytes fused to donor nuclei which are

capable of proliferation in an undifferentiated state after continuous culture;

(c) primate embryonic stem cells of the above type wherein the cells can differentiate into cells derived from mesoderm, endoderm, and ectoderm germ layers when the cells are injected into an immunocompromised mouse; and

(d) methods to produce such stem cells.

These and still other objects and advantages of the present invention will be apparent from the description which follows. The following description is merely of the preferred embodiments. The claims should therefore be looked to in order to understand the full scope of the invention.

DETAILED DESCRIPTION OF THE INVENTION

A. Derivation From Oocytes

Oocyte Collection And Activation

Rhesus (or other primate) oocytes can be obtained from hyperstimulated primates by laparoscopy according to routine methods (R. Schramm *et al.*, 11 Hum. Reprod. 1690-1697 (1996); R. Schramm *et al.*, 11 Hum. Reprod. 1698-1702 (1996); D. Wolf *et al.*, 43 Mol. Reprod. Dev. 76-81 (1996); D. Wolf *et al.*, 27 Mol. Reprod. Dev. 261-280 (1990)) and cultured in modified CMRL-1066 (D. Boatman, *The Mammalian Preimplantation Embryo*, Plenum Press, New York, 273-308 (1987)) until metaphase II (MII) stage.

Primate oocytes can then be activated by known osmotic (J. Levron *et al.*, 3 Zygote 157-161 (1995)), electrical (V. Marshall *et al.*, 9 Abstr. Serv. 98 J. Reprod. Fertil. (1992)), chemical (6-DMAP/ionomycin, SR²⁺ or cyclohexamide - G. Wu, 55 Biol. Reprod. 260-270

(1996); L. Meng *et al.*, 57 Biol. Reprod. 454-459 (1997); T. Wakayama *et al.*, 394 Nature 369 (1998)), or biological (injection of sperm factor - H. Wu *et al.*, 46 Mol. Reprod. Dev. 176-189 (1997) and H. Wu *et al.*, 49 Mol. Reprod. Dev. 37-47 (1998)) methods. Activation can occur prior to, concomitant with, or after karyoplast fusion. See I. Wilmut *et al.*, 385 Nature 810-813 (1997).

Donor Cell Preparation

Fibroblasts or other donor cell types can be prepared from fetal or postnatal tissues (e.g. skin biopsy) by mechanical disruption followed by trypsinization and cultured in DMEM-10% Fetal Calf Serum (FCS). See generally E. Robertson, *Teratocarcinoma And Embryonic Stem Cells: A Practical Approach*, IRL Press, Washington, D.C. 71-112 (1987).

Immediately prior to nuclear transfer, cell cultures can be trypsinized, washed, and resuspended in transfer medium (TALP-Hepes). See generally B. Bavister *et al.*, 28 Biol. Reprod. 983-999 (1983).

Oocyte Enucleation And Nuclear Transfer

For 30 minutes prior to enucleation, MII oocytes are proposed to be incubated in transfer medium containing the cytoskeletal inhibitor cytochalasin B (7.5 µg/ml). See generally L. Meng *et al.*, 57 Biol. Reprod. 454-459 (1997).

Enucleation is then proposed to be accomplished with a beveled micropipette (J. McGrath *et al.*, 228 Exper. Zoo. 355-362 (1983) and J. McGrath *et al.* 220 Science 1300-1302 (1983), and enucleation confirmed by staining of the removed karyoplast with the dye Hoechst 33342 (3 µg/ml) and direct observation under epifluorescence. See

generally S. Stice *et al.*, 54 Biol. Reprod. 100-110 (1996). A single donor cell is then proposed to be selected with the micropipette and injected under the zona pellucida.

5 Fusion

Fusion of the donor cell and oocyte cytoplasm are proposed to be accomplished by viral (J. McGrath *et al.*, 228 Exper. Zoo. 355-362 (1983) and J. McGrath *et al.* 220 Science 1300-1302 (1983), chemical (M. Sims *et al.*, 91 P.N.A.S. USA 6143-6173 (1994), or electrical methods (L. Meng *et al.*, 57 Biol. Reprod. 454-459 (1997).

10 Alternatively, nuclei are proposed to be injected directly into the cytoplasm using techniques analogous to the injection techniques described in T. Wakayama *et al.*, 15 394 Nature 369 (1998)

Culture And ES Cell Derivation

The nuclear transfer products are proposed to be cultured to the blastocyst stage in standard medium used in human IVF clinics, such as S1/S2 or G1.2/G2.2 medium (Scandinavian IVF Science AB, Gothenburg, Sweden). The inner cell mass of the resulting blastocyst should then be removed by immunosurgery (D. Solter *et al.*, 72 P.N.A.S. USA 5099-5102 (1975)), and cultured on mitotically inactivated fibroblasts. After approximately 25 9-15 days, the resulting mass of cells should then be dissociated, replated on fibroblasts, and ES cells selected by their characteristic morphology and expanded. See J. Thomson *et al.*, 38 Curr. Top. Dev. Biol. 133-165 (1998), J. Thomson *et al.*, 106 AMPHIS 149-157 (1998), and 30 U.S. Serial No. 08/591,246, filed January 18, 1996.

B. Nuclear Transfer Directly To ES Cell Cytoplasm

In the next protocols we propose the approaches of using ES cells (or preferably ES cell cytoplasts) to reprogram adult nuclei. As a first experiment, we
5 suggest that rhesus ES cells (XX karyotype) be transfected stably with the hygromycin resistance cassette, and primary embryonic fibroblasts (an XY karyotype) be transfected stably with a neomycin resistance cassette.

10 The rhesus ES cells and fibroblasts containing these markers are then proposed to be fused by electrofusion or by other chemical fusion methods. See generally N. Duzgunes 220 Methods Enzymol. (1993), and heterokaryons selected for hygromycin/neomycin resistance. The
15 resulting clones are proposed to be expanded, karyotyped, injected into SCID mice, and analyzed for contributions to derivatives of all three embryonic germ layers. This will determine whether undifferentiated ES cells can reprogram differentiated nuclei using this technique to
20 create tetraploid heterokaryons.

We next propose that rhesus ES cell cytoplasts and neomycin resistant fibroblast karyoplasts be prepared and fused by similar methods. See generally N. Duzgunes, 220 Methods Enzymol. (1993). The fusion products will be
25 plated on neomycin resistant primary fibroblasts under G418 selection, and colonies with a ES cell morphology expanded and examined for developmental potential.

C. Demonstration Of ES cell Pluripotency

ES cells can be characterized by their ability to
30 differentiate to derivatives of all three embryonic germ layers, endoderm, mesoderm, and ectoderm even after

prolonged culture. This is demonstrated either by letting the cells over-grow and pile up in culture, when spontaneous differentiation occurs, or by injecting into immunocompromised mice, where teratomas form with cells representing all three germ layers.

5 D. Application

The present invention is primarily intended to be used for therapeutic purposes. For example, a cell (e.g. a skin fibroblast or other cell) can be taken from a patient having a disease (e.g. juvenile onset diabetes).
10 One can then reprogram the differentiated nucleus by transfer to an enucleated oocyte, culture the nuclear transfer product to a blastocyst stage, derive undifferentiated ES cells from the inner cell mass,
15 differentiate the ES cells (e.g. to pancreatic β -cells), and use those cells for transplantation therapy to treat the diabetes.

Similar approaches should be of value in connection with treatment of other diseases such as Parkinson's, Huntington's, Alzheimer's, ALS, spinal cord injuries, multiple sclerosis, muscular dystrophy, liver disease, heart disease, cartilage deficiency, burns, and cancer, and for other therapeutic purposes. In each case where a supply of cells is needed, the invention is intended to provide them at low cost and with reduced rejection risk.
20
25

The present invention is also designed to provide cells useful in studying early development genes and for other research purposes. It will likely also be useful in providing cells suitable for diagnostic purposes. See
30 generally the applications described in WO 98/07841.

Industrial Applicability

The invention is designed to provide precursors for developing transplantable cells. These cells are intended to be useful for therapeutic and other purposes.

CLAIMS

I claim:

1. A primate embryonic stem cell that is MHC-compatible to a selected donor, wherein the cell has been derived by transplantation of cell nucleus from a differentiated cell of the donor to an enucleated primate oocyte.
5
2. The cell of claim 1, wherein the cell is a human embryonic stem cell that has been derived from a human oocyte and a nucleus from a human somatic cell or human germ cell.
3. The cell of claim 1, wherein the cell is genetically identical with respect to all nuclear genes to the selected donor.

4. A purified preparation of primate embryonic stem cells which (i) is capable of proliferation in an in vitro culture, (ii) maintains the potential to differentiate to derivatives of endoderm, mesoderm, and 5 ectoderm tissues, (iii) is inhibited from differentiation when cultured on a fibroblast feeder layer, and (iv) has genes coding for a major histocompatibility complex identical to a major histocompatibility complex of a specified nuclear donor.

5. The preparation of claim 4, wherein the cells are genetically identical for all nuclear genes to the specified nuclear donor.

6. The preparation of claim 4 wherein the cells were derived from ES cell cytoplasts fused to karyoplasts.

7. A method for producing the preparation of claim
4, comprising transplanting cell nucleus from a
differentiated cell of a primate donor to an enucleated
primate oocyte so as to provide embryonic stem cells
5 coding for said donor's major histocompatibility complex.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09784

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 //C12N5/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 30683 A (UNIV MASSACHUSETTS A PUBLIC IN) 16 July 1998 (1998-07-16) page 11, line 9 - page 12, line 22; claims 1,8,11 ---	1-7
X	DATABASE WPI Section Ch, Week 8847 Derwent Publications Ltd., London, GB; Class D16, AN 88-334664 XP002114590 & JP 63 248385 A (YOSHIDA K), 14 October 1988 (1988-10-14) abstract ---	1-3
A	WO 98 07841 A (UNIV MASSACHUSETTS) 26 February 1998 (1998-02-26) cited in the application page 6, line 22 - page 8, line 22 ---	4-7
X	WO 98 07841 A (UNIV MASSACHUSETTS) 26 February 1998 (1998-02-26) cited in the application page 6, line 22 - page 8, line 22 ---	4-6
A	---	1-3,7
	---	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09784

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 22362 A (WISCONSIN ALUMNI RES FOUND) 25 July 1996 (1996-07-25) cited in the application abstract; claim 1 page 7, line 9 - page 8, line 6 ----	4,5
A	WO 95 03398 A (UNIV MONASH ;TATHAM BRENDAN GEORGE (AU)) 2 February 1995 (1995-02-02) page 1, line 1 - line 5; claim 21; example 1 ----	1-7
P,X	DE 197 09 549 A (BORN EBERHARD PROF DR) 24 September 1998 (1998-09-24) the whole document -----	1-7